Article

Molecular identification of *Fusarium* spp. isolated maize in Sinaloa, Mexico

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Abstract

Fusarium species cause macular diseases in the maize that induce rotting of stems, roots and grains of corn and finally the death of the plant. In the present study, the presence and diversity of *Fusarium* species associated with these damages in Sinaloa was molecularly identified. During the sowing periods in the years 2013-2014, stem and root tissue from young and adult plants, as well as diseased pod kernels, was collected in the municipalities of Ahome, Culiacan and Elota in the state of Sinaloa. In this investigation, species *F. verticillioides*, *F. oxysporum*, *F. subglutinans*, *F. equiseti*, *F. nygamai*, *F.* cf. *bullatum* and *F. andiyazi*, were identified by enzymatic sequencing analysis, where *F. verticilloides* was the most predominant species. Likewise, the presence in Mexico of *Fusarium* cf. *bullatum* causing damage to corn.

Keywords: *Zea mays* L., *Fusaria*, enzymatic sequencing.

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Introduction

Mexico is the center of origin and diversity of maize (*Zea mays* L.), which has given rise to a greater number of endemic maize races, wild varieties and genotypes Center for Studies for Change in the Mexican Countryside (Ceecam, 2012). In Latin America, about 220 maize races have been described (Goodman and Bird, 1977), of which 64 have been identified and described mostly for Mexico (Anderson, 1946; Hernández and Alanis, 1970; Sánchez *et al*., 2000).

Mexican maize races have been grouped, based on morphological, adaptation and genetic characters (isoenzymes) into seven racial groups or complexes (Goodman and Bird, 1977; Sánchez *et al*., 2000). INIFAP and its predecessors since 1942 have developed and released more than 250 corn varieties and hybrids, which in some cases have not been sufficient and it should be recognized that in general research, from its inception, has privileged agriculture with the greatest productive potential (Espinosa *et al*., 2009). The cultivation of corn occupied the first place by area planted in Mexico with 7.6 million hectares, and a total production of 19 504 050 t (SIAP, 2015).

All the entities of the country present some level of production of this crop; however, seven concentrate 64.5% of the volume, being the state of Sinaloa the main producer with 20.7% in a harvested area of 359 400 ha (SIAP, 2015). Among the most economically important diseases are those caused by the genus *Fusarium* sp. (Hernández *et al*., 2016) since it is a microorganism with a great diversity of species, special forms and races, associated with plant diseases (Leslie and Summerell, 2006). The phytopathogenic species of this genus constitute a group of filamentous fungi that are widely distributed in the soil and that colonize the aerial and subterranean parts of a wide range of plant species, generating symptoms of yellowness, wilting, root rot, cankers and finally the death of many crops (Leslie and Summerell, 2006).

In several countries, a great diversity of *Fusarium* species has been found infecting plants (Mesterhaz *et al*., 2012). In Mexico, the diversity of *Fusarium* species that generate decay in commercial and native maize populations has been documented (Morales *et al*., 2007; García and Martínez, 2010; Leyva *et al*., 2014; Briones *et al*., 2015).

In addition to their phytopathogenic behavior, species of the genus *Fusarium*, in their infection process, synthesize secondary metabolites called mycotoxins, within which have been described those of the zearalenone group, trichothecenes and fumonisins (Eckard *et al*., 2011), which are high toxicity and that generate human and animal disturbances in health.

There are reports that *F. solani* (Martius) Apple & Wollenweber emend. Snyder & Hansen (teleomorph= *Nectria haematococca* (Berkeley & Broome) Samuel & Niberberg), *F. oxysporum* Schlechten emend. Snyder & Hansen (without sexual reproduction) and *F. verticilloides* (Saccardo) Nirenberg (formerly called *F. moniliforme* (teleomorph= *Gibberella moniliformis* Wineland) are the species that mainly cause harm to humans (Tezcan *et al*., 2009: de Souza *et al*., 2014), however, it was recently diagnosed with *F. subglutinans* Wollenber & Reiking Nelson, Toussoun & Marasas (teleomorph= *Gibberella subglutinans* Nelson, Toussoun & Marasas (Campos *et al*., 2013) and *F. napiforme* Marasas, Nelson & Rabie (without sexual reproduction) that cause mycosis in humans (de Souza *et al*., 2014) and damage grasses (Leslie and Sumerell, 2006).

Fusarium proliferatum (Matsushima) Nirenberg (teleomorph= *Gibberella intermedia* (Kuhlman) Samuels, Nirenberg & Seifert), *F. subglutinans* and *F. verticillioides* are the most well-known species as causal agents of corn root and ear damage worldwide (Bertechini *et al*., 2012; Kaur*et al*., 2014), in Mexico, *F. verticillioides* and *F. subglutinans* (Morales *et al*., 2007; Figueroa *et al*., 2010; López *et al*., 2014) have been the most reported in this disease.

The genus *Fusarium* is also known for its taxonomic difficulties in the definition and identification of species at the morphological level, hence the relevance of integrating other elements such as pathogenic and molecular characterization that have also proved to be reliable in the evaluation of genetic diversity within this organism (Bacon *et al*., 1994). Currently molecular biology techniques based on PCR with the use of specific primers and molecular markers have been used for the identification of *Fusarium* species. The use of the partial gene of calmodulin has been reported for the identification of *F. verticillioides*, *F. proliferatum* and *F. subglutinans* (Mule *et al*., 2004).

Another alternative is the gene of elongation factor 1 alpha (EF-1α or TEF), which codes for an essential protein of the translation machinery. Its phylogenetic utility lies in the fact that its sequence is highly conserved at the genus *Fusarium* level, for which primers have been designed that represent a better opportunity to separate species, whose amplified products generate a ~700 bp fragment flanking 3 introns (O'Donell *et al*., 1998; Geiser *et al*., 2004). On the other hand, the ITS (Lin *et al*., 2014), the RAPDs (Kaur*et al*., 2014) and the RFLPs (Hsuan *et al*., 2010) are also frequently used as molecular tools for the identification of *Fusarium* species. The objective of the present study was to know the *Fusaria* species that cause diseases in the cultivation of corn, in sowings of irrigation of the autumn-winter cycle in the state of Sinaloa, Mexico.

Materials and methods

The work was carried out during the spring-summer, autumn-winter 2012-2013 and 2013-2014 agricultural cycles, collecting seedlings, adult plants and corn cobs in different agricultural sites in the municipalities of Ahome, Culiacan and Elota in the state of Sinaloa (Figure 1). The laboratory research was carried out in the Biotechnology Unit of the Valle de Culiacan Experimental Field of the National Institute of Forestry, Agriculture and Livestock Research (INIFAP), 24° 37' 59.3" north latitude, 107° 26' 31.0" west longitude 54 meters above sea level, Culiacán, Sinaloa.

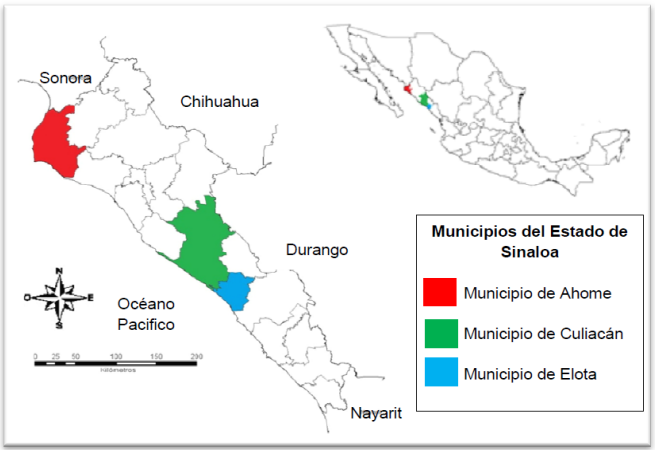

Figure 1. Location of the study area.

Isolation of monosporic cultures from *Fusaria*

Seed tissues of seedlings and maize plants collected were cut into 3 mm longitudinal pieces in addition to grain of corn cobs, which were superficially disinfested with 2% sodium hypochlorite for 2 min and 70% ethanol for 2 min, followed by three consecutive washes with sterile distilled water. For the growth and development of the fungus, the plant tissue was placed in Petri dishes with potato-dextrose-agar culture medium (PDA-Difco) supplemented with 1.5 mL L^{-1} of PCNB (pentachloronitrobenzene) and cefuroxime (200 mg L^{-1}), incubated at a temperature of 25 °C for five days. The identification of the fungus was carried out in a compound microscope (Olympus Cx31), based on the morphology of the mycelium, microconidia and macroconidia proposed by Leslie and Summerell, (2006).

To obtain monosporic cultures of *Fusaria*, a small fragment of the mycelium grown in PDA medium was cut out, which contained the fungicide PCNB and cefuroxime, resuspended in 1 mL of sterile distilled water and the number of conidia in serial dilutions was counted by a hematocytometer (Hernández and Rangel, 2011), from these were obtained aliquots that were distributed on PDA medium in Petri dishes, from where the monosporic culture was obtained. The growth of the fungus was observed under the compound microscope, placing individual germinated spores, which were selected and transferred individually to a new Petri dish with PDA medium supplemented with PCNB and cefuroxime. These were incubated under the same conditions mentioned above. A small piece of PDA medium with the developed fungus was transferred to test tubes containing sterile sieved sand (8 x 10 threads cm^{-3}) with Komada liquid medium (Komada, 1975) and stored in a refrigerator at a temperature of 4 °C for its preservation and later use.

Extraction of DNA from monosporic cultures

For DNA extraction, the previously described method was used (Velarde *et al*., 2015), for which the mushroom mycelium was obtained by scraping with a sterile bacteriological handle from the solid medium, this was placed in porcelain mortar and pistil, previously sterilized for 5 min in a microwave oven household appliance (LG, model MS-1446SQP/01), operating at microwave frequency, around 2.45 GHz (GigaHertz) and finally cooled to -70 ºC. Then, 1 mL of extraction buffer containing: 30 mM NaCl, 30 mM ethylenedinitrilotetraacetic acid (EDTA) and 250 mM Tris Base (pH 8.5) was added, with which it was macerated. The maceration product was placed in 1.5 mL Eppendorf tubes with their respective labeling. Then, 100 L of 10% ammonium cetyltrimethylbromide (CTAB) and 250 L of 5M Sodium Chloride (NaCl) were added to the sample, being incubated at 95 °C for 10 min and subsequently centrifuged at 12 000 rpm for 10 min; After this time, the aqueous solution (supernatant) was transferred to a new tube. A volume of cold chloroform (v/v) was added to the aqueous solution and it was stirred in a vortex unit for a few seconds and then centrifuged at 10 000 rpm for 5 min. After this time, the aqueous solution was transferred to a new tube, adding a volume of cold absolute isopropanol and stirring manually.

Subsequently, the samples were stored for one hour at -20 °C to allow DNA precipitation. After this time, the samples in tubes were centrifuged at 12 000 rpm for 10 min to obtain the DNA pellet, which was allowed to dry at laboratory temperature for two hours, finally, the DNA obtained from the different isolates was resuspended in 50 L of nuclease-free water (Promega) and stored at 4 °C for its conservation.

Analysis by PCR

A group of primer pairs were used for PCR analysis of *Fusaria* DNA, which were originally described to separate species-specific and in our case, we used them to generate first-hand information for epidemiological studies on the incidence of *Fusaria*: the pair FOF1/FOR1 for *F. oxysporum* (Mishra *et al*., 2003), VER1/2, PRO1/2 and SUB1/2 for species-specific such as *Fusarium verticillioides*, *F. proliferatum* and *F. subglutinans* (Mule *et al*., 2004) respectively (Table 1). A thermal cycler (Nyx Technik Amplitronyx series 6 A6 (ATC401) Thermal Cycler) was used for the reaction. The final reaction mixture (15 µL) contained 100 ng of DNA, an equimolar mixture of dATP, dCTP, dGTP and dTTP, MgCl2, PCR buffer, DNA *Taq* polymerase (provided by Promega® PCR Master Mix, Catalog No. M7502), 40 pmoles of each oligonucleotide (Sigma®).

Table 1. Specific initiators, used for the amplification of predicted fragments for Fusaria species.

Initiator's name	Sequences of initiators 5'→3'	Specific species
SUB1	CTGTCGCTAAACCTCTTTATCCA	*F. subglutinans* [a]
SUB2	CAGTATGGACGTTGGTATTATATCTAA	
PRO1	CTTTCCGCCAAGTTTCTTC	*F. proliferatum* [a]
PRO2	TGTCAGTAACTCGACGTTGTTG	
VER1	CTTCCTGCGATGTTTCTCC	*F. verticillioides* [a]
VER2	AATTGGCCATTGGTATTATATATCTA	
FOF	ACATACCACTTGTTGCCTCG	*F. oxysporum* [b]
FOR	CGCCAATCAATTTGAGGAACG	
EF1	ATGGGTAAGGA(A/G)GACAAGAC	*Fusaria* [c, d]
EF2	GGA(G/A)GTACCAGT(G/C)ATCATGTT	

[a]= Mule *et al*. (2004); [b]= Mishra *et al*. (2003); [c]= O'Donnell *et al*. (1998); [d]= Geiser *et al*. (2004).

The amplification conditions for *F. oxysporum* were: 1 cycle at 95 °C, 5 min; 30 cycles (95 ºC, 1 min, 53 ºC, 1 min, 72 ºC, 1 min) and a final extension cycle at 72 ºC, 10 min and 4 ºC, while for *F. verticillioides*, *F. proliferatum* and *F. subglutinans* consisted of: 1 cycle at 95 ºC, 5 min; 30 cycles (95 ºC, 1 min, 56 ºC, 1 min, 72 ºC, 1 min), a final extension cycle at 72 ºC, 10 min and 4 ºC. The amplified products were analyzed on 1% agarose gels, stained with a Gel Red solution (Biotium, catalog No. 41003).

In order to confirm the identity of the species detected with the set of primers described above, a second PCR analysis was performed in which the pair of primers corresponding to the TEF gene region were selected, for which DNAs from 33 strains monosporic were selected. The primer pairs EF1 and EF2 (O'Donnell *et al*., 1998; Geiser *et al*., 2004) (Table 1) were used with an alignment temperature of 55 °C.

Enzymatic sequencing

Only the amplified products of the TEF gene region were excised from the agarose and purified through silica columns (EZ-10 Spin Column DNA Gel Extraction Kit BS354, Bio Basic Inc.). Once the purified PCR fragment was obtained, these samples were sent for sequencing to the National Laboratory of Genomics for Biodiversity (LANGEBIO) of the Center for Research and Advanced Studies (CINVESTAV-IPN), Unit Irapuato, Guanajuato, Mexico, based on the ddNTPs method (Sanger *et al*., 1977), using a 3730 XL DNA sequencer (Applied Biosystems, Foster City, CA) and the Big DyeTerminator 3.1 kit (Applied Biosystems, Foster City, CA). The search for similarity between DNA sequences was made through the BLAST program, with which the nucleotide sequences under study were compared with the databases of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/), identifying the homology values.

Postulate of Koch de *Fusarium* cf. *bullatum*

The seeds of the Aperlado creole corn were obtained from the maize germplasm bank of the Valle de Culiacan Experimental Field-INIFAP. For the preparation of the inoculum of the fungus (accession number in GenBank-NCBI, KR612339), it was grown in PDA culture medium supplemented with PCNB (1.5 mL L^{-1}) and cefuroxime (200 mg L^{-1}) for one week, 25 ºC. From this medium, eight fragments of mycelium were cut and deposited in a flask with AMA medium (sand 450 g, corn flour 50 g, sterile water 50 mL) incubated under the same conditions for 15 days. Disinfestation of maize seeds was washed in a 3% sodium hypochlorite solution for 3 min and then rinsed in sterile distilled water and for germination they were deposited in an incubator at 25 °C for 48 h.

Germinated seeds, these were selected for uniformity in size, depositing one seedling per pot. For the preparation of the soil-substrate, a mixture of dead soil (1.5 kg), peat (0.5 kg) and half AMA (0.250 kg) (with inoculum of the fungus) was made and 600 g of this mixture were deposited in pots previously sterilized. The inoculum quantity for this species was 5 000 ufc g^{-1} of soil. The experiment was carried out based on the protocol of Trapero and Jimenez (1985) and Navas *et al*. (2007) in a greenhouse under controlled conditions at a temperature of 25 ±3 °C and a photoperiod of 12 h, for 60 days. Two repetitions of pots were performed, including the controls without inoculum.

Phylogenetic analysis

The alignment of the nucleotide sequences of the elongation factor EF-1α of *Fusarium* species was performed with the Clustal W method and the dendrogram was constructed with the Máxima Parsimonia method using the SPR algorithm (Nei and Kumar, 2000) and a bootstrap of 1 000 replicas (Felsenstein, 1985). All analyzes were performed with the MEGA program version 6.0 (Tamura *et al*., 2013).

Results and discussion

Molecular identification of *Fusarium* spp by PCR

We obtained 116 monosporic cultures, 25 seedling isolates, 36 adult plant and 55 ear, all with rot damage to which the genus *Fusarium* was identified by the morphology of bicellular microconidia, septate and canoe macroconidia. and the septate mycelium, according to the keys proposed by Leslie and Summerell (2006) (Figure 2).

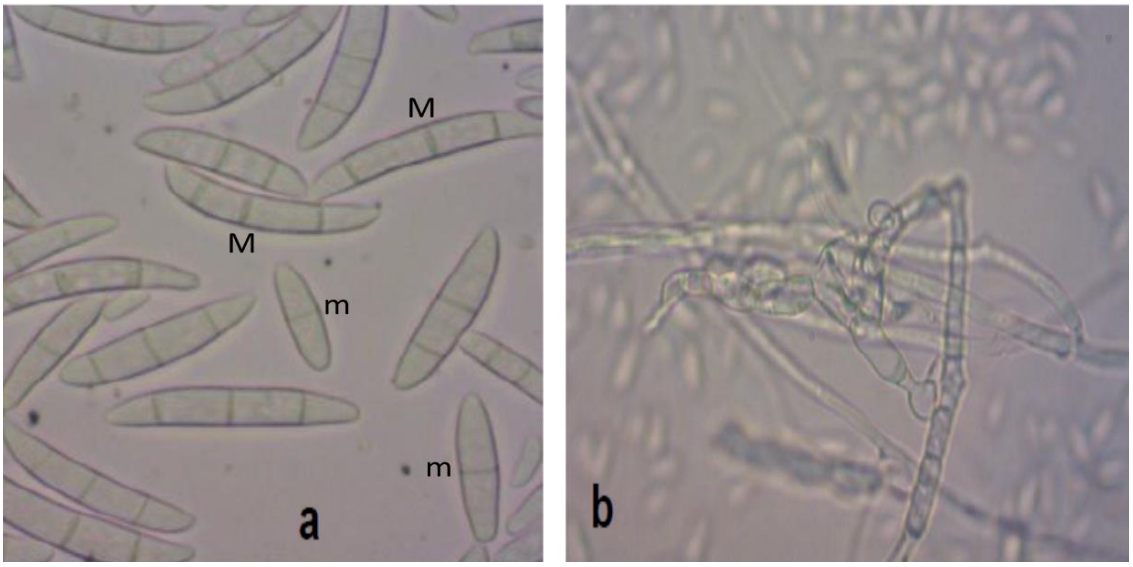

Figure 2. *Fusarium* morphology: a) microconidia (m), macroconidia (M); b) mycelium.

To each monosporic isolate the DNA was extracted for specific amplification by PCR with the use of primers described for the species: *F. oxysporum*, *F. verticilloides*, *F. subglutinans*y *F. proliferatum* (Table 1). Of the 116 samples that were morphologically identified as *Fusarium* spp., 35 (30.17%) amplified the predicted fragment of 350 bp, for *F. oxysporum* (Figure 3), a species that has been mentioned causing vascular damage in this crop in the state of Puebla, Mexico (García and Martínez, 2010).

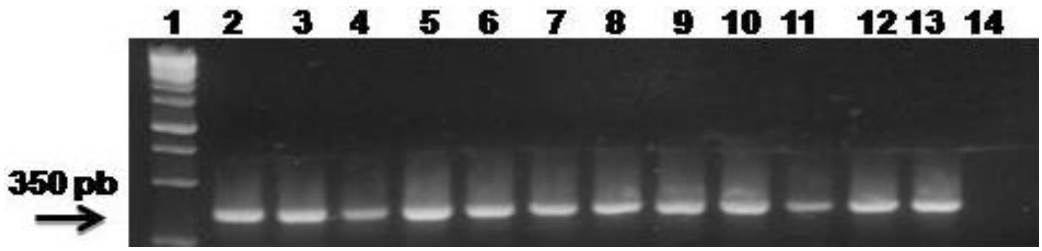

Figure 3. Electrophoretic analysis in 1% agarose. Amplification of the 350 bp fragment by PCR with FOF and FOR specific primers to detect *F. oxysporum*. Lanes: 1= Gene Ruler 1Kb DNA Ladder; 2-12= *Fusarium* DNA obtained from sick corn collected in different localities of the state of Sinaloa; 13= positive control (DNA of *F. oxysporum*) and 14= negative control (without DNA).

Of the DNA extracted from the total monosporic decepas, 70 samples amplified with the primers described for *F. verticilloides* (60.34%), this species presenting the highest frequency of amplifications in this study, confirming its prevalence worldwide (Gimeno and Martins, 2011). Recent studies indicate that *F. verticilloides* is the main species causing damage to root and stem rot in corn in the state of Sinaloa (Lopez *et al*., 2014). In Figure 4, the amplified fragments with a predicted size of 578 bp described for the species *F. verticilloides* are observed (Table 1).

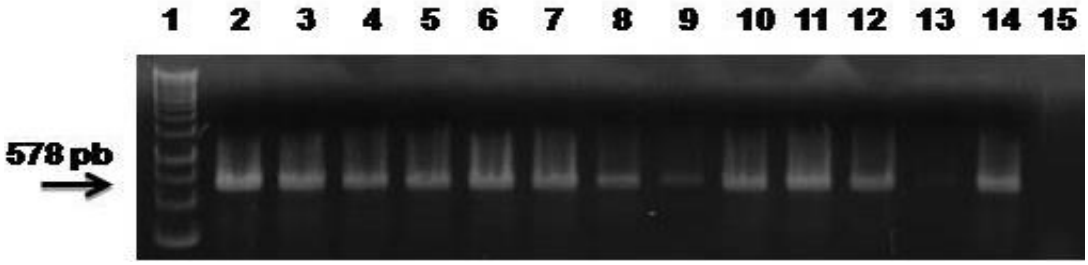

Figure 4. Electrophoretic analysis in 1% agarose. Amplification of the 578 bp fragment by PCR with specific VER1 and VER2 primers to detect *F. verticilloides*. 1= Gene Ruler 1Kb DNA Ladder, 2-13= *Fusarium* DNA obtained from sick corn collected in localities of the state of Sinaloa, 14= positive control (*F. verticilloides* DNA) and 15= negative control (without DNA).

Only three of the 116 samples analyzed amplified with the primers corresponding to *F. subglutinans* (2.59%). In Figure 5, the amplified fragments with a predicted size of 631 bp are observed. Worldwide, this species after *F. verticilloides* is the second causal agent of corn root rot (Gimeno and Martins, 2011). In Mexico, *F. subglutinans* has been found in the state of Guanajuato (Figueroa *et al*., 2010) and in the State of Mexico (Morales *et al*., 2007; Rivas *et al*., 2011).

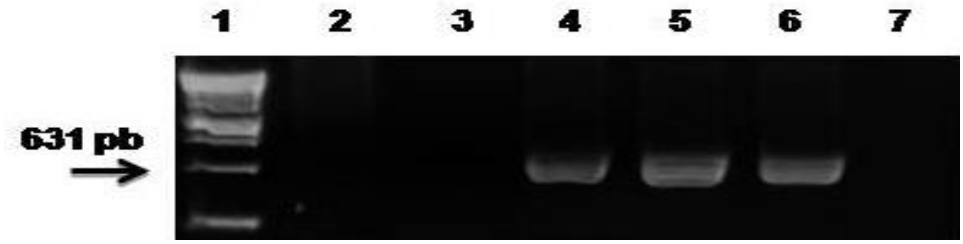

Figure 5. Electrophoretic analysis in 1% agarose. Amplification of the 631 pb fragment by PCR with specific SUB1 and SUB2 primers to detect *F. subglutinans*. 1= Gene Ruler 1Kb DNA Ladder; 2-6= *Fusarium* DNA obtained from samples of sick corn collected in localities of the state of Sinaloa, 6= positive control (DNA of *F. subglutinans*) and 7= negative control (without DNA).

Regarding the detection with the specific primers of *F. proliferatum*, no amplifications were observed in the eight DNA samples analyzed, which were amplified with the TEF primers and included among the 33 sequenced samples.

Enzymatic sequencing

Of the 116 amplifications that were obtained in total, 32 fragments were sequenced. The identity of the sequences in our study showed a high similarity for seven different species. Table 2 shows the accession numbers in the NCBI, tissue where they were isolated, location and the percentage

of homology. For this, each of our accessions was compared with the database of the National Center for Biotechnology Information (NCBI-USA), which generated the homology values with each accession described for the different species, from which the identity of: *F. verticilloides*, *F. oxysporum*, *F. subglutinans*, *F. nygamai* Burgess &Trimboli (teleomorph: *Gibberella nygamai* Klaasen & Nelson), *F. andiyazi* Marasas, Rheeder, Lamprecht, Zeller & Leslie (without sexual reproduction), *F.* cf. *bullatum* and *F. equiseti* (Corda) Saccardo (teleomorph: *Gibberella intricans* Wollenweber) whose accession numbers are described in Table 2.

Table 2. Identity of *Fusarium* species based on the sequencing of the TEF (EF-1α) gene.

Strain	Location	Tissue	Accession to GenBank	Association based on the sequence	Identity (%)	Comparative accession of GenBank
526MC	Ahome	Cob	JN806238	*F. verticilloides*	100%	JF740729
527MC	Ahome	Cob	JN806239	*F. verticilloides*	100%	JF740729
528MC	Ahome	Cob	JN806240	*F. verticilloides*	100%	JF740729
529MC	Ahome	Cob	JN806241	*F. verticilloides*	100%	JF740729
530MC	Ahome	Cob	JN806242	*F. verticilloides*	100%	JF740729
532MC	Ahome	Cob	JN806243	*F. verticilloides*	100%	JF740729
533MC	Ahome	Cob	JN806244	*F. verticilloides*	100%	JF740729
536MC	Ahome	Cob	JN806245	*F. verticilloides*	100%	JF740729
538MC	Ahome	Cob	JN806246	*F. verticilloides*	100%	JF740729
610CS	Culiacán	Seedling	KR905566	*F. verticilloides*	100%	JF740729
613CUL	Culiacán	Seedling	KF753752	*F. cf. bullatum*	99%	JX268977
622CS	Culiacán	Seedling	KR905551	*F. verticilloides*	100%	JF740729
623MS	Ahome	Seedling	KR905567	*F. cf. bullatum*	100%	JX268977
627CS	Culiacán	Seedling	KR905552	*F. verticilloides*	100%	JF740729
630CS	Culiacán	Seedling	KR905553	*F. verticilloides*	100%	JF740729
635CS	Culiacán	Seedling	KR905554	*F. verticilloides*	100%	JF740729
638ES	Elota	Seedling	KR905555	*F. verticilloides*	100%	JF740729
646ES	Elota	Seedling	KR905556	*F. verticilloides*	100%	JF740729
649ES	Elota	Seedling	KR905557	*F. verticilloides*	100%	JF740729
652MS	Ahome	Seedling	KR905558	*F. nygamai*	100%	JF740790
655ES	Elota	Adult plant	KR905559	*F. verticilloides*	100%	JF740729
663ES	Elota	Adult plant	KR905560	*F. verticilloides*	100%	JF740729
676ES	Elota	Adult plant	KR905562	*F. verticilloides*	100%	JF740729
677CS	Culiacán	Adult plant	KR905563	*F. verticilloides*	100%	JF740729
678CS	Culiacán	Adult plant	KR612341	*F. nygamai*	100%	JF740790
680MS	Ahome	Adult plant	KR706385	*F. equiseti*	91%	KF514661
681CS	Culiacán	Seedling	KR706384	*F. equiseti*	91%	KF514661
706ES	Elota	Seedling	KR612339	*F. cf. bullatum*	100%	JX268977
771CS	Culiacán	Adult plant	KR706383	*F. subglutinans*	99%	DQ837698
777ES	Elota	Adult plant	KR612338	*F. andiyazi*	99%	KM462947
789ES	Elota	Adult plant	KR905564	*F. oxysporum*	99%	KM092371
843CS	Culiacán	Adult plant	KR612340	*F. oxysporum*	100%	DQ435354

The species *F. equiseti* has been identified in the Bajio area, Guanajuato, Mexico (Figueroa *et al*., 2010); however, Leslie and Summerell (2006) state that the species is considered a saprophyte or secondary invader; however, Madania *et al*. (2013) has reported it in Syria causing damage to corn plants. As for *F. nygamai*, it has been identified in Sinaloa worldwide causing damage (Leyva *et al*., 2014), which was identified in this study.

With regard to *F. andiyazi*, it has been described with cob rot damage in China (Zhang *et al*., 2014), Syria (Madania *et al*., 2013) and also in Sinaloa, Mexico (Leyva *et al*., 2014). Finally, for *F.* cf. *bullatum* there is a report in Iran that reports its isolation and damage in maize (Rahjoo *et al*., 2008).

Postulate of Koch de *Fusarium* cf. *bullatum*

After 60 days of inoculation under controlled conditions, it was observed that creole corn was damaged and root rot was damaged by *Fusarium* cf. *bullatum* (Fig. 6), then monosporic strains were isolated and morphologically observed macroconidia (4-5-septa) (Figure 7 a, m), microconidia (1-2 septa) (Figure 7 a, m) and phialides on carnation leaf agar (CLA) (Figure 7-b). Later, a strain was asylated by DNA and through the TEF gene it was identified molecularly that it is *Fusarium* cf. *bullatum*. Enzymatic sequencing was recorded in the NCBI gene bank (GenBank-NCBI-USA) with accession number KX545253.

Figure 6. Koch postulate of *Fusarium* cf. *bullatum* showing damage to the maize Aperlado creole under controlled conditions in the greenhouse.

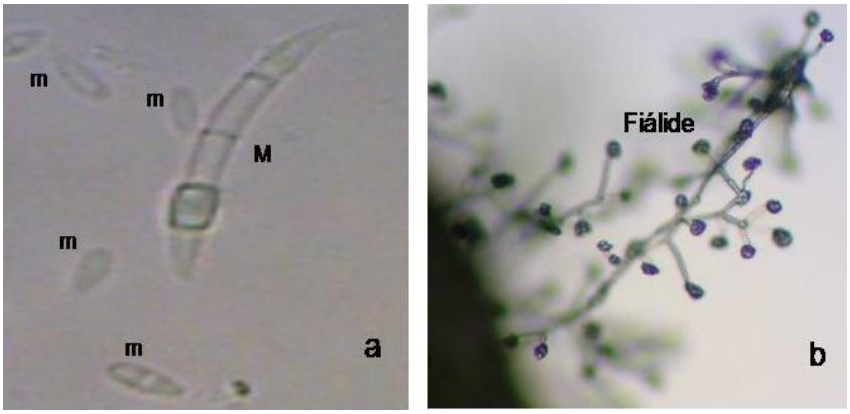

Figure 7. *Fusarium* cf. *bullatum* morphology: a) microconias (m); macroconidia (M); and b) phialides.

Phylogenetic analysis

The phylogenetic relationship of Maximum Parsimony of the elongation factor EF-1α of *Fusarium* species was analyzed in 32 sequences with 587 positions using the model of nucleotide replacement SPR (pruning and reconnection of a subtree), which better adjusted to EF-1α. The analysis of the sequences generated 8 trees of maximum parsimony, with consistency indexes of 83.5% and retention of 92%, both indices of wide reliability in phylogenetic comparisons (Nei and Kumar 2000). The consensus of these trees retains a total of 9 clades.

According to the *Fusarium* mating population (Summerell and Leslie, 2006), *F. verticillioides* and *F. andiyazi*, synonyms of *Gibberella fujikuroi* belong to mating population A, coincidentally, they were part of the same group in our dendogram (Figure 6). In the 99% cluster in which *F. oxysporum*, *F. nygamai* and *F. subglutinans* are found, which have previously been described within the mating population G (Summerell and Leslie, 2006); in this analysis, this grouping among these species was also reflected in the dendrogram (Figure 6).

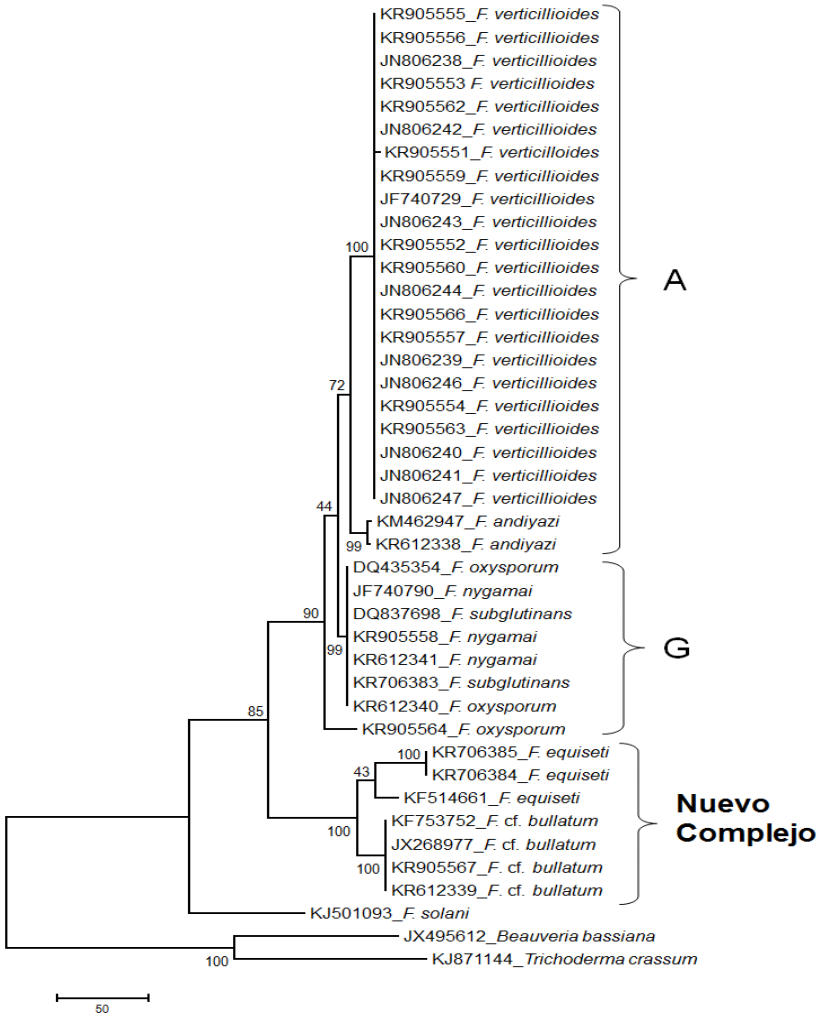

Figure 8. Maximum Parsimony Dendrogram based on sequences of the EF-1α elongation factor of *Fusarium* species. The dendrogram was constructed with 32 nucleotide sequences, analyzing 587 positions and a Felsenstein index of 1 000 replicas.

The cluster where *F. equiseti* and *F. cf. bullatum*, is separated from the mating population A and G, which was recently described as a new complex (Castella and Cabañes, 2014); however, Summerell and Leslie (2006) mention as *F. equiseti* var *bullatum* within the same species.

Conclusions

The distinction of species within the *Fusarium* genus using morphological characters was not precise.

The use of specific primers used for the identification of *Fusaria* species in order to know, first hand, the incidence of each species detected, gave results coinciding with the sequencing of the species: *F. verticilloides*, *F. subglutinans* and *F. oxysporum.*

With the use of the TEF primers, it was possible to confirm the identity and presence of seven species of *Fusaria* isolated in the maize: *F. verticilloides*, *F. subglutinans*, *F. oxysporum*, *F. equiseti*, *F. nygamai*, *F. andiyazi* and *F.* cf. *bullatum*.

F. verticilloides was the predominant species with 60.34%, *F. oxysporum* was the species that occupied the second place, with an incidence of 30.17%, *F. subglutinans* and *F.* cf. *bullatum* in third place with an incidence of 2.59%.

According to our results, the close phylogenetic relationship between the species *F. verticillioides* and *F. andiyazi* coincided with the mating population A described by Summerell and Leslie (2006), as well as *F. oxysporum*, *F. nygamai* and *F. subglutinans*, within of mating population G and for the case of *F. equiseti* and *F.* cf. *bullatum* that formed a separate group, were associated with a new complex described by Castella and Cabañes, (2014).

This research reports for the first time the presence in Mexico of *Fusarium* cf. *bullatum* causing damage to corn.

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